# SPECTROPHOTOMETRIC DETERMINATION OF FLURORAL AND URANINE IN MILK


## SUMMARY

To render antibiotic-containing milk easily recognizable, it has been suggested that a dye marker be added to veterinary antibiotic preparations which are to be infused into the udder. Ideally, the dye would be eliminated from the udder at the same rate as the antibiotic, and the milk should be discarded as long as a trace of dye was detected. A mixture of two fluorescein dyes, (Fluroral and Uranine), has shown promise for this purpose. To properly evaluate such a procedure, quantitative methods for measuring the marker content of milk were needed. Therefore, simple and rapid spectrophotometric methods have been devised for the quantitative determination of fat-soluble fluorescein (fluroral) and water-soluble fluorescein (uranine) in milk. Reproducibility values ranged from 92 to 100%, and recovery values from 94 to 103%.


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Recent work by Hargrove, Lehman, and Matthews (1) and Hargrove, Plowman, and Wright (2) has established the feasibility of detecting antibiotics in milk by incorporating fluorescent materials in antibiotic preparations intended for intramammary infusion. Best results were obtained by adding a mixture of fluroral (Fluroral 7GA,[1, 2] oil-soluble fluorescein) and uranine (disodium fluorescein) to the antibiotic preparations. These authors established the presence of the dyes by examining the milk under ultraviolet illumination and obtained a semiquantitative estimate of their concentration by diluting the milk until the fluorescence could no longer be seen. For laboratory and statistical purposes a precise numerical measurement of the concentration of each dye in a sample of milk was desired. The following simple and rapid methods were evolved to fill this need.

The use of dye markers to detect antibiotics in milk has not been approved by regulatory agencies. It would be advantageous, however, to have suitable procedures and analytical methods available, in the event that they are approved and used in veterinary antibiotic preparations. The principles developed in this study may be useful, even though a different marker is eventually employed.

## APPARATUS AND REAGENTS

Spectrophotometer, Beckman Model B or similar instrument.

Shaking machine, reciprocating type, capable of agitating extraction flasks about 400 times per minute.

Sodium hydroxide solution, about 1 *N*.

Ethyl ether, U.S.P. or better.

Received for publication January 8, 1960.

[1] E. H. Sargent and Company, Chicago, Illinois.

[2] The use of trade names is for the purpose of identification only, and does not imply endorsement of the product or its manufacturer by the U. S. Department of Agriculture.

Sodium tungstate solution, dissolve 1 lb. C.P. $Na_2WO_4 \cdot 2H_2O$ in one liter of water.

Sulfuric acid solution, 50% by volume.

Standard dye solutions, 50 mg. of uranine are weighed into a 500-ml. volumetric flask and made up to volume with 1 *N* NaOH. Fifty milligrams of fluroral are weighed into a 500-ml. volumetric flask and made up to volume with ethyl ether. Each solution contains 100 $\mu$g. of dye per milliliter.

## EXPERIMENTAL PROCEDURE

*Preparation of standard curves.* Using the standard dye solutions, dilutions are prepared so that 1 ml. of solution will contain 0.5, 1.0, 2.0, 5.0, and 10.0 $\mu$g. of dye. The color of the uranine dilutions is measured at the wave length of maximum absorption (480 m$\mu$), using 1 *N* NaOH as the reference solution. The color of the fluroral dilutions is measured at the wave length of maximum absorption (420 m$\mu$), using ethyl ether as the reference solution. Both curves follow Beer's law over the range of concentrations used here. Micrograms of dye per milliliter of solution are plotted against the absorbance readings.

*Presence of fluroral and uranine.* The presence of fluorescent dyes is ascertained by examining the milk under ultraviolet illumination.

*Blank value.* A sample of milk known to be free of the dyes is treated in the same maner as the regular samples, so that an appropriate blank correction can be made.

*Preparation of milk sample.* A representative portion of milk (about 50 ml.) is warmed in a 45–50° C. water bath with occasional shaking for 15 min., or until sample is uniform in appearance.

*Determination of uranine.* Fifty-five milliliters of 1 *N* NaOH are placed in a 125-ml. Erlenmeyer flask. Exactly 5.0 ml. of the warmed milk sample are pipetted into the NaOH, and mixed thoroughly by quickly rotating the flask 40–50 times. The mixture is allowed to stand for 5 min., until the precipitate has clumped into large particles. The solution is filtered through a lint-free fluted filter paper and the filtrate is collected in a 100-ml. volumetric flask. The Erlenmeyer flask is rinsed with 40 ml. of 1 *N* NaOH and the washings poured through the filter. The filter paper is washed with 1 *N* NaOH from a wash bottle until the filtrate is exactly 100 ml. The volumetric flask is stoppered and the contents mixed thoroughly. Since the color will darken on standing, the color is measured in the spectrophotometer within 0.5 hr. at 480 m$\mu$, using 1 *N* NaOH as the reference solution. The absorbance reading is corrected for the effect of the blank and the micrograms of uranine per milliliter of solution are obtained from the standard curve. This value is then converted into micrograms of uranine per milliliter of original milk.

*Determination of fluroral.* Five milliliters of the well-mixed milk sample are placed in a 125-ml. Erlenmeyer flask (or suitable shaking flask). Then 0.5-ml. sodium tungstate solution and 0.5-ml. sulfuric acid solution are added and all mixed thoroughly. Forty milliliters of ether are added and the flask is tightly stoppered with a clean rubber stopper. The flask is placed in the

shaker and shaken for 3 min. A 100-mm. funnel is prepared for filtering the ether solution by tamping a small plug of pyrex wool into the upper end of the funnel stem. Filter paper is not used, since it absorbs too much of the dye. A 100-ml. volumetric flask is placed under the funnel. The pressure which builds up in the extraction flask is released cautiously and away from the operator, as the curd is dangerously acidic. The ether is decanted into the funnel, leaving all curd in the flask. As much ether as possible is expressed by carefully jarring the curd to the bottom of the flask. This ether is decanted into the funnel also. The shaking-extracting-filtering procedure is repeated three more times, using 30 ml. of ether each time. The last 30-ml. portion of ether, after extraction, is usually colorless to the eye. If color is apparent it indicates that the sample size was too large. In this event the determination is repeated, using a 2.50-ml. sample of milk, 0.25 ml. sodium tungstate solution, and 0.25 ml. sulfuric acid solution. Less ether will also be needed with a smaller sample, 40 ml. for the first extraction and 25 ml. for each of the three remaining extractions. Regardless of the sample size, exactly 100 ml. of ether filtrate is collected, the volumetric flask is stoppered, and the contents mixed thoroughly. The color is measured in the spectrophotometer at 420 m$\mu$, using ether as the reference solution. The absorbance reading is corrected for the effect of the blank, and the micrograms of fluroral per milliliter of solution are obtained from the standard curve. This value is then converted into micrograms of fluroral per milliliter of original milk.

## DISCUSSION

The problem involved in this study was one of separating quantitatively from milk a water-soluble compound (uranine) and a fat-soluble compound (fluroral). Although chromatographic and fluorometric methods were tried, the simple colorimetric method described here proved to be the most rapid and satisfactory.

*Uranine determination.* To separate the uranine from the insoluble constituents of milk, sodium hydroxide was chosen as the precipitating agent because the yellow-green color of uranine is most intense in an alkaline solution; however, the strength of the alkaline solution is not critical. To show this, equal amounts of uranine were dissolved in equal amounts of 0.5 *N*, 1.0 *N*, and 2.0 *N* NaOH. The absorbance readings of these solutions were the same, .136 in 0.5 *N*, .135 in 1.0 *N*, and .136 in the 2.0 *N* solution. Also, the precipitation of the milk proteins and the liberation of the uranine from milk is not dependent upon a specific normality of the NaOH. Milk samples containing 250 $\mu$g. of uranine were analyzed, using 0.5 *N*, 1.0 *N*, and 2.0 *N* NaOH as the precipitating agent. In each case, quantitative recovery of the 250 $\mu$g. of uranine was obtained.

It is important to allow enough time for the precipitate to form into large aggregates; otherwise, some cloudiness may pass through the filter paper. Fresh milk samples are more likely to give a cloudy filtrate than milk samples stored for some time. If a cloudy filtrate is encountered, it can be satisfactorily clarified by centrifuging.

Throughout the uranine determination the fat-soluble fluroral is trapped,

along with the fat, in the precipitate and does not pass through the filter paper.

The increase in color intensity obtained by allowing the alkaline solution of uranine to stand is not the result of any change in the uranine but is due to the action of the alkali on the lactose present. This color, which is also yellow-green, can be easily demonstrated by placing a few crystals of lactose in some 1 *N* or 2 *N* NaOH and allowing it to stand for some time. Although the blank correction should allow for this change in color, it is best to measure the uranine color as soon as possible.

Duplicate uranine determinations usually agree within 5% of each other, except in samples of very low concentration, and the method can detect 1 μg. of uranine per milliliter of milk with ease. Recovery data for the uranine determination are given in Table 1.

TABLE 1
Recovery of uranine and fluroral added to milk

| Uranine *(μg.)* | | |
|---|---|---|
| Added | Recovered | % Recovered |
| 41 | 42 | 102 |
| 100 | 97 | 97 |
| 150 | 141 | 94 |
| 250 | 257 | 103 |
| 400 | 380 | 95 |
| Fluroral *(μg.)* | | |
| 200 | 200 | 100 |
| 250 | 240 | 96 |
| 300 | 310 | 103 |
| 400 | 390 | 97 |
| 500 | 480 | 96 |

*Fluroral determination.* In analyzing for fluroral, a curd was desired which would bind up all the water and uranine present but allow the fat-soluble fluroral to be extracted with ether. The sodium tungstate-sulfuric acid curd is a gelatinous curd having these properties. Shaking must be vigorous, however, to allow the ether to penetrate into the curd and extract the fluroral. This curd also has no tendency to form an emulsion, which is always a problem when extracting milk with ether. The four portions of ether used to extract the fluroral (40-30-30-30) make a total of 130 ml., but only 100 ml. of ether are desired in the filtrate. Normal evaporation which will occur during the filtration process usually reduces the 130 ml. to the desired 100 ml.

The preparation of the milk before pipetting the fluroral sample should be thorough, since fluroral has a tendency to clump with the fat and cling to the sides of the container. Warming the milk and mixing completely is a necessity.

Duplicate fluroral determinations usually agree within 8% of each other, except in samples of very low concentration, and the method can detect 2 μg. of fluroral per milliliter of milk without difficulty. Recovery data for the fluroral determination are given in Table 1.

## APPLICATION OF THE METHODS

Analyses were made on milk obtained from udder quarters which had

received infusions of antibiotic preparations containing 125 mg. of each dye. Infusions were made after milking and the collection of milk samples for analysis was started the next milking. Details of the procedure are covered in the article by Hargrove, Plowman, and Wright (2). Table 2 shows the decrease

TABLE 2

Amount of fluroral and uranine in successive milkings after infusion

| Milking | Fluroral | | Uranine | |
|---|---|---|---|---|
| | Absorbance | Micrograms per milliliter of milk | Absorbance | Micrograms per milliliter of milk |
| 1 | 1.066 | 510.0 | 1.162 | 144.4 |
| | 1.020 | 490.0 | 1.178 | 146.0 |
| 2 | .222 | 106.0 | .147 | 18.0 |
| | .215 | 102.6 | .160 | 19.6 |
| 3 | .040 | 17.8 | .050 | 6.2 |
| | .041 | 18.2 | .048 | 6.0 |
| 4 | .032 | 14.2 | .049 | 6.0 |
| | .030 | 13.4 | .053 | 6.6 |
| 5 | .018 | 8.0 | .037 | 4.6 |
| | .016 | 7.2 | .040 | 5.0 |
| 6 | .008 | 3.6 | .045 | 5.6 |
| | .006 | 2.6 | .034 | 4.2 |
| 7 | .004 | 1.8 | .026 | 3.2 |
| | .004 | 1.8 | .030 | 3.8 |

in dye content for seven milkings after infusion, the range of concentrations which were encountered, and the agreement between duplicate determinations.

## REFERENCES


(1) Hargrove, R. E., Lehman, R. J., and Matthews, C. A. The Use of Fluorescent Materials for the Indirect Detection of Antibiotics. J. Dairy Sci., 41: 617. 1958.

(2) Hargrove, R. E., Plowman, R. D., and Wright, W. W. Use of Markers in Veterinary Preparations for the Detection of Antibiotics in Milk. J. Dairy Sci., 42: 202. 1959.